Universidade de Aveiro Departamento de Biologia
2018

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O efeito da disponibilidade de azoto no conteúdo em antioxidantes de duas espécies halófitas em hidroponia de água salgada

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Aplicada, realizada sob a orientação científica da Doutora Ana Isabel Lillebø Batista, Investigadora Principal do Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

Halimione portulacoides, *Chenopodium quinoa*, biorremediação, metabolitos secundários, eutrofização, aquacultura sustentável.

resumo

O desenvolvimento sustentável da aquacultura requer a procura de melhores práticas e soluções alternativas de forma a minimizar os problemas (ambientais e económicos) causados pelos efluentes gerados. Estes são ricos em nutrientes, não podendo ser diretamente descartados no ambiente, uma vez que podem causar eutrofização. Os produtores primários têm sido usados para a remoção do excesso de nutrientes inorgânicos dissolvidos, atuando como espécies extrativas na biorremediação de efluentes. No contexto da aquacultura marinha, a biorremediação de efluentes salinos implica a utilização de espécies tolerantes à salinidade, como é o caso das plantas halófitas. Tendo em conta a atual agenda de economia circular, é dada preferência a espécies de plantas com potencial comercial. Neste enquadramento, o objetivo do presente estudo foi testar o efeito da disponibilidade de azoto (N) na produção de metabolitos secundários nos produtores primários *Halimione portulacoides* e *Chenopodium quinoa*, como indicadores da saúde da planta quando cultivada em hidroponia.

Halimione portulacoides é uma halófita que apresenta potencial para remoção de nutrientes de efluentes salinos e *Chenopodium quinoa* é uma halófita facultativa muito estudada pelo seu valor nutricional e tolerância à salinidade, mas não como agente de biorremediação.

A experiência foi realizada numa estufa com condições controladas onde as plantas foram cultivadas numa solução hidropónica com salinidade de 20 e quatro concentrações de azoto: 20 mg l^{-1}; 40 mg l^{-1}; 100 mg l^{-1} e 200 mg l^{-1}. Após 5 semanas (*H. portulacoides*) e 4 semanas (*C. quinoa*), as plantas foram caracterizadas tendo em conta o ganho de biomassa e o conteúdo em clorofila, ORAC, fenóis totais, flavonoides totais, ácido ascórbico, N-C total, e ainda o conteúdo em elementos tidos como relevantes. Foram determinadas as concentrações de nitrato e de fosfato na solução hidropónica e efetuado o cálculo do balanço de massa.

Os resultados revelaram que ambas as espécies produziram um elevado teor em antioxidantes, quando cultivadas em concentrações de azoto de 20 mg l^{-1}. Essas plantas apresentaram um baixo incremento em biomassa, indicando uma resposta de stress. Os resultados indicam que a concentração que despoleta essas condições de stress encontra-se entre 40 mg l^{-1} e 20 mg l^{-1}. Quando cultivadas em concentrações de azoto iguais ou superiores a 100 mg l^{-1}, as plantas apresentaram maior incremento em biomassa e menor conteúdo em antioxidantes. Resultado particularmente relevante na espécie *C. quinoa*, que apresentou os valores mais elevados para estes parâmetros. Relativamente à remoção de nutrientes da solução hidropónica, *C. quinoa* demonstrou maior capacidade de extração quando comparada com *H. portulacoides*, mesmo com o aumento de disponibilidade de azoto.

Tendo em conta a gama de concentrações de azoto testadas, este estudo revela o potencial de integração de *H. portulacoides* e de *C. quinoa* como espécies extrativas em sistemas de água salgada (salinidade de 20), em diferentes regimes de produção aquícola, que podem ir desde o cultivo semi-intensivo ao super-intensivo.

keywords

Halimione portulacoides, *Chenopodium quinoa*, bioremediation, secondary metabolites, eutrophication, sustainable aquaculture.

abstract

The sustainable development of aquaculture requires the search for best practices and alternative solutions to reduce constrains (environmental and economic) posed by the generated effluents. These are nutrient rich and cannot be directly discharged into the environment, as it might induce eutrophication. Primary producers have been used for the uptake of the excess of dissolved inorganic nutrients, acting as extractive species for bioremediation of effluents. In the context of marine aquaculture, bioremediation of saline effluents implies the use of salt tolerant species, like halophytes. In light of the current circular economy agenda, preference is given to potentially commercial plant species. In frame of the above mentioned, the objective of this study was to test the effect of nitrogen (N) availability in the production of secondary metabolites of primary producers *Halimione portulacoides* and *Chenopodium quinoa*. The secondary metabolites were used as proxy for plant health status, when cultivated under hydroponic conditions.

Halimione portulacoides is a halophyte with potential for the removal of nutrients from marine effluents. *Chenopodium quinoa* is a facultative halophyte that has been subject of several studies in terms of nutritional value and salt tolerance but not as an agent for bioremediation.

The experiment was performed under controlled greenhouse conditions where plants were cultivated in hydroponic solution with a salinity of 20 and four concentrations of nitrogen: 20 mg l^{-1}; 40 mg l^{-1}; 100 mg l^{-1} and 200 mg l^{-1}. After 5 weeks (*H. portulacoides*) and 4 weeks (*C. quinoa*), plants were characterized considering the biomass gain, the content in chlorophyll, ORAC, total phenols, total flavonoids, ascorbate, total N-C, and the content in relevant elements. The concentration of nitrate and phosphate in the hydroponic solution was analysed for mass balance calculation.

Results showed that both species produced high antioxidant content when cultivated in hydroponic solution with 20 mg l^{-1} of nitrogen concentrations, but at the cost of biomass gain, thus indicating a stress response. Results also indicate that the concentration that triggers the stress conditions range between 40 mg l^{-1} and 20 mg l^{-1}. When cultivated in hydroponic solution with 100 mg l^{-1} of nitrogen concentrations or higher concentrations, plants presented higher biomass gain and lower antioxidant content. This is particularly relevant for *C. quinoa* which presented the higher values for these parameters. Regarding the removal of nutrients from the hydroponic solution, *C. quinoa* presented higher extraction capacity, when compared to *H. portulacoides.* This is still true with increasing concentrations of nitrogen. Considering the range of nitrogen concentrations tested, this study shows the potential of *H. portulacoides* and *C. quinoa* to be integrated as extractive species in marine aquaculture facilities (salinity of 20). This can be done under different production regimes, from semi-intensive to super-intensive aquaculture systems.

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1. Introduction

1.1. Aquaculture in the context of Blue Growth strategy

On a global society facing climate change, competing for natural resources and where human population is expected to reach over 9 billion people by 2050 (FAO, 2018), finding new sources of food and sustainable sources of production is of major interest. Marine and freshwater ecosystems are pillar sources of food and livelihood for people around the world and proper governance of these resources is challenging.

In 2007, a discussion meeting between the Food and Agriculture Organization of the United Nations (FAO) and aquaculture experts resulted in the proposition of an Ecosystem Approach to Aquaculture (EAA), one of the first steps to move aquaculture development towards greater sustainability as it follows three principles, being the first one "aquaculture should be developed in the context of ecosystem functions and services with no degradation of these beyond their resilience capacity" (Soto et al., 2008).

Later, in 2012, FAO launched the Blue Growth Initiative that intends to promote responsible aquaculture practices, considering better fishing policies and ocean governance. In line with the United Nations, the European Commission (EC) developed the Blue Growth Strategy with the intent to foster innovative technologies and investment in promising maritime sectors. In the "Strategic Guidelines for the sustainable development of EU aquaculture" document, the EC recognizes that the success of aquaculture is dependent on the good quality of marine and fresh waters (European Union: European Commission, 2013). This EC communication also transmits the implementation of new policies to encourage the development of European aquaculture by simplifying administrative procedures, coordinate spatial planning particularly for coastal aquaculture, produce marketing plans to enhance competitiveness and promote collective management to ensure high quality products.

In the particular case of Portugal, the National Ocean Strategy 2013-2020 was adopted to allow the country to meet the challenges for the development of its maritime economy and the strategic framework at international level. Hence, the Strategic Plan for Portuguese Aquaculture 2014-2020 was established with the intent to increase and diversify aquaculture products, considering sustainability and food security guidelines and contribute to the creation of jobs and development of the industry.

1.1.1. The need for sustainable aquaculture

With the world's capture fisheries stagnated due to overexploited fish stocks and regulatory measures, aquaculture seems to be the most reliable solution for the ever increasing demand for seafood for human consumption and other industries. Rapidly growing, the sector was responsible for almost 80 million tons of fish and 30.1 million tonnes of aquatic plants (mostly seaweeds) produced in 2016 (FAO, 2018). This intensification often generates environmental threats, being one of the major concerns the degradation of aquatic ecosystems. European environmental legislation, like the Water Framework Directive (WFD) (Directive 2000/60/EC) and the Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC) regulates the quality of the discharged effluents in European industry. However, in Asian countries this is still a problem (Luo et al., 2018; Park et al., 2018).

Untreated aquaculture effluents carrying uneaten feeds, metabolic wastes, pesticides, antibiotics and other contaminants are often discharged to downstream water bodies (Granada et al., 2016). This nutrient enrichment causes the deterioration of aquatic and benthic systems by increasing nitrogen and phosphorus concentrations in the water leading to eutrophication (Granada et al., 2016).

To address this problem recirculating aquaculture systems (RAS) were developed in the last decades, where water is partially reused after physical, chemical and biological treatment (Piedrahita, 2003). RAS systems are usually implemented for intensive and super-intensive

productions to offset the high cost of waste treatment due to the low energy efficiency, since these systems consume much more electricity than most conventional systems (Ayer and Tyedmers, 2009).

Thus, the improvement of aquaculture waste management is of paramount importance to decrease both environmental and economic impacts caused by nutrient pollution and the need for water renewal.

The initiatives developed by governmental agencies worldwide aim at stimulate the industry to follow a more sustainable approach through optimization of the methods of production.

1.1.2. IMTA as an opportunity in circular economy

In the last two decades Integrated Multi-Trophic Aquaculture (IMTA) appeared as a more sustainable solution aiming to: (i) diversify production, (ii) make it cost-effective and (iii) reduce the environmental impacts. IMTA follows a more sustainable approach, as it is ecosystem based. It combines the culture of fed species (usually finfish or shrimp) with the culture of organisms that can extract the particulate organic matter (filter-feeding and detritivore organisms) and dissolved matter (plants and seaweeds) originated in fed trophic levels. In these systems, the most used organisms to remove the dissolved nutrients are seaweeds and among the organisms that remove particulate organic matter, the most common are bivalves, but also polychaetes and sea cucumbers (Custódio et al., 2017; Granada et al., 2016; Marques et al., 2018). IMTA can be applied in both fresh and marine water on land, coastal or off-shore facilities (Barrington et al., 2009; Troell, 2009). The use of organisms from different trophic levels helps to reduce nutrient load in the effluents and provides economic diversification (Chopin, 2006). In fact, when compared to other systems, IMTA appears to present economic benefits because it creates profitability from nutrients that would otherwise be wasted and allows the diversification of species in the same

production (Klinger and Naylor, 2012), creating a loop production and placing aquaculture in the circular economy market.

Another approach to integrated aquaculture is aquaponics. Aquaponics systems integrate exclusively hydroponic cultivation of primary producers with recirculating aquaculture (RAS). In this way, the dissolved nutrients wasted from the fish is used as an input for plant growth (fig. 1) (Goddek et al., 2015). Similar to RAS, aquaponics systems require a solids removal treatment like settling ponds or mechanical filters and a biofilter for nitrification processes before the effluent reaches the hydroponic unit (fig. 2) (Rakocy et al., 2006). Thus, these systems still involve some significant costs with waste removal. Amongst the most profitable vegetable species used in this systems are lettuce, aromatic herbs and watercress and the most commonly cultured fish species is tilapia (Rakocy et al., 2006), all of them produced in freshwater.

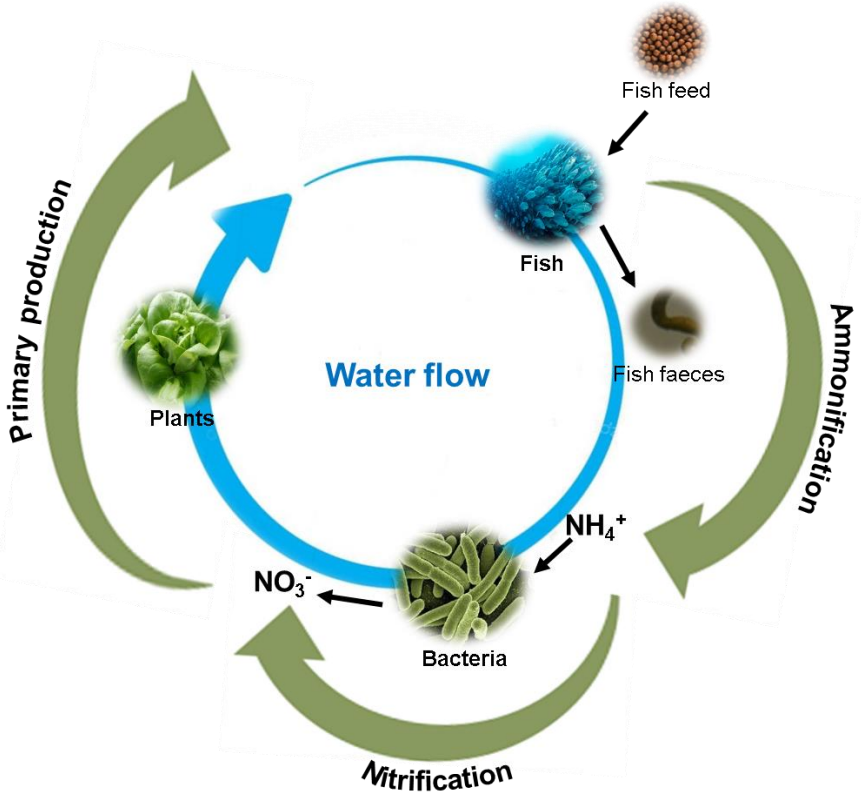

Figure 1. Nitrogen cycle and biological relationship in an aquaponics system. Adapted from Goddek et al., 2015.

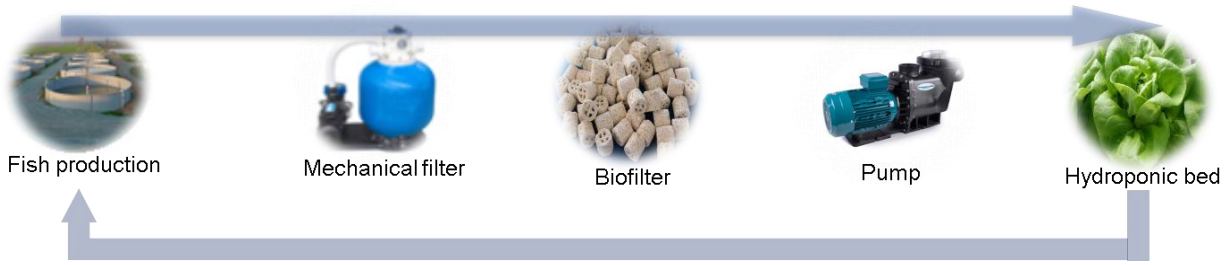

Figure 2. Aquaponics system components. Adapted from Goddek et al., 2015.

The previous examples demonstrate how the aquaculture sector can decrease its environmental impacts and expand and diversify production by investing in innovative technologies and approaches.

As the majority of European aquaculture is marine and coastal based (FAO, 2018), the need for remediation solutions for saline effluents is imperative.

Salt tolerant plants have started to receive some attention in the last years due to their potential applicability in bioremediation of marine aquaculture effluents, even in IMTA and aquaponics systems (e.g. Custódio et al., 2017).

1.2.Salt tolerant plants

Salt tolerant plants can grow and complete their life cycle with high concentrations of salt in their environment. Also designated as halophytes, they can be classified as obligate or facultative halophytes depending on their salt-tolerating capacity. Obligate halophytes fail to survive under fresh water conditions whereas facultative halophytes grow well with freshwater but can also withstand some concentrations of salinity (Krauss and Ball, 2013).

Under culture conditions, halophytes can tolerate NaCl concentrations up to 500mM (Flowers et al., 1977) but, because in their natural environments conditions are very complex, a general definition sets a minimum tolerance of 200mM salt concentration to complete their life cycle, provided that the environmental conditions are within the natural

range (Flowers et al., 1986). Halophytes can be found in a variety of environments like sand dunes, rocky coasts, inland deserts and salt marshes (Flowers et al., 1986). In order to sustain these high concentrations of salinity they acquired specific physiological adaptations like water retention, protection against ROS and ion transportation (Flowers and Colmer, 2015).

1.2.1. Potential for bioremediation of marine effluents and integration in IMTA systems

Salinity is a stress factor for many plant species and might affect photosynthesis and other essential metabolic processes and ultimately will inhibit plant growth (Parida and Das, 2005). Therefore, nutrient uptake by the plant may depend on the salt tolerance of the species. Different plant species also present different optimal concentrations for nutrient requirements and uptake efficiency, so the nutrients availability might also influence the filtering effect (Buhmann and Papenbrock, 2013b).

Due to their tolerance to elevated concentrations of NaCl, halophytes can be used as an alternative cash crop for regions of the globe where soil salinization and scarcity of fresh water are a problem (Ventura and Sagi, 2013), but it also confirms their potential applicability in remediating saline effluents (Buhmann and Papenbrock, 2013a; Custódio et al., 2017; Marques et al., 2017).

Buhmann et al. (2015) evaluated culturing conditions for the use of halophytes as biofilter in saline effluents and concluded that hydroponic culture might be the best mode of culture for halophytes, in terms of production of valuable biomass and nutrient recycling, when compared with sand and expanded clay. They also showed that species like *Tripolium pannonicum* (Jacq.) Dobrocz. (Homotypic synonym for *Aster tripolium*), *Plantago coronopus* L., *Atriplex halimus* L., *Atriplex portulacoides* L. (Homotypic synonym for *Halimione portulacoides*) and *Lepidium latifolium* L. are suitable for the production of valuable biomass at a salinity of 15.

Other experimental studies with *Sesuvium portulacastrum* L. and *Batis maritima* L. presented a daily nitrogen removal up to 67% (Boxman et al., 2017) and dissolved inorganic nitrogen decrease over 60% with *Halimione portulacoides* (L.) Aellen (Marques et al., 2017).

1.2.2. Secondary metabolites and applications

Environmental stresses like water scarcity, high salinity, extreme luminosity or temperature and nutrient deficiency leads to the formation of reactive oxygen species (ROS) that can interfere with DNA, lipids and proteins which triggers oxidative stress in plants (Gill and Tuteja, 2010). To protect themselves against these toxic compounds, plants hold antioxidant defence systems. Halophytes possess an efficient antioxidant system with several enzymatic and non-enzymatic compounds. Among the non-enzymatic there is polyphenols (which include flavonoids), vitamins and carotenoids (Ksouri et al., 2008).

Phenolic compounds have also been associated with several health benefits in humans due to their anti-carcinogenic, anti-inflammatory and anti-aging activities (Ksouri et al., 2012; Middleton, 1998; Pandey and Rizvi, 2009). Thus, these compounds can help prevent cardiovascular disorders (Rodriguez-Mateos et al., 2014). Another relevant example is ascorbic acid (vitamin C). Humans are not able to produce ascorbic acid on their own, so they must obtain it from external sources like fruits and vegetables (Padayatty et al., 2003). Ascorbic acid is essential for collagen, neurotransmitters and carnitine biosynthesis (Naidu, 2003).

Other authors summarize the potential value of halophyte's secondary metabolites to nutraceuticals, pharmacognosy and food applications (Buhmann and Papenbrock, 2013b; Ksouri et al., 2012). Extracts of halophytes have been used in traditional medicine for the treatment of diabetes, cancer and inflammation, for example (Buhmann and Papenbrock, 2013b).

More recently, (Lopes et al., 2016) revealed the potential applicability of several halophyte species from the South Coast of Portugal in cosmetic and pharmaceutical industries due to their high antioxidant content.

Hereupon, more research is necessary to really understand the full potential of these plants for remediation of saline effluents and integration in complex aquaculture systems, by seeking new techniques and trying out new species in a consumer demanding and competitive economy market.

1.2.3. Halimione portulacoides

Halimione portulacoides (fig. 3) is a perennial, C3 shrub with a height ranging from 20-50cm that colonizes the lower and mid marshes across the coasts of Europe, North Africa and South-West Asia (Chapman, 1950). Because it is found mainly on salt-marshes with frequent tidal inundations, it can sustain salinities similar to that of seawater (Carvalho et al., 2001).

Until now, most of the studies about *H. portulacoides* have focused on the influence of salinity on growth (Jensen, 1985; Redondo-Gómez et al., 2007), in antioxidant capacity and in secondary metabolites production (Boestfleisch et al., 2014; Boestfleisch and Papenbrock, 2017). In Portugal the few studies about *H. portulacoides* are dedicated to its chemical composition (Neves et al., 2007; Rodrigues et al., 2014; Vilela et al., 2014) and remediation potential (Marques et al., 2017).

Figure 3. *Halimione portulacoides*. Source: MBA research group, University of Aveiro .

1.2.4. Chenopodium quinoa

Chenopodium quinoa Willd., commonly known as Quinoa (fig. 4) is a salt tolerant plant native from the Andean region, capable of growing at different altitudes, from sea level to high mountains and at different environmental conditions from cold to highland and tropical environments (Jacobsen, 2003; Nowak et al., 2016). Its grains have been consumed for thousands of years and are known for their high nutritional content, classifying this plant as a pseudocereal (Nowak et al., 2016). This functional food has a high protein content, essential amino acids and also important fatty acids and minerals (Vega-Gálvez et al., 2010). Quinoa seeds also have a high antioxidant content, namely bioactive flavonoids, when compared with other cereals (Hirose et al., 2010; Vega-Gálvez et al., 2010). Due to its worldwide agriculture potential and excellent nutritional value this plant is classified by FAO as one of humanity's promising crops that can contribute to the food security of this century (FAO Regional Office for Latin America and the Caribbean and PROINPA, 2011).

This facultative halophyte can tolerate salinities similar to that of seawater (Hariadi et al., 2011; Turcios et al., 2016) and has been the subject of studies to understand its resistance mechanisms to abiotic stress (Adolf et al., 2013; Jacobsen et al., 2007, 2003), crop potential (Jacobsen, 2003; Repo-Carrasco et al., 2003; Vega-Gálvez et al., 2010) and even its biogas potential (Turcios et al., 2016).

Figure 4. *Chenopodium quinoa*. Source: www.aphotoflora.com.

1.3. Objectives

Since both above mentioned species present good nutritional value and tolerance to high salt concentration, they show potential to be successfully cultivated under saltwater hydroponics and be integrated in marine and coastal IMTA systems. In aquaculture, one of the main constituents of the effluents is nitrogen (N), which, in the form of nitrate, is essential (limiting nutrient) for vegetable crop cultivation (Rakocy et al., 2006). To the author's best knowledge there are no previous studies on the influence of nitrogen availability in the production of secondary metabolites, namely antioxidants, in these plant species when produced under hydroponic conditions. The objective of this study was to

test the effect of nitrogen (N) availability in the production of secondary metabolites, as a proxy for cultured halophytes health status. The tested null hypothesis was: N availability does not affect the production of secondary metabolites in *Halimione portulacoides* (L.) Aellen and in *Chenopodium quinoa* Willd. var. Titicaca cultured under saltwater hydroponics conditions.

2. Methodology

2.1. Experimental set-up

Cuttings of *H. portulacoides* obtained from a mature plant collected at the North Sea, Jade Bay, Germany, were planted in individual pots with propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany) and grown for 4 weeks and then placed in hydroponics.

Seeds of *C. quinoa* (obtained from Sven-Erik Jacobsen, University of Copenhagen, Denmark, but originating in Peru, close to lake Titicaca) were sown in propagation soil (Einheitserde, Einheitserdewerk Hameln-Tündern, Germany) and watered with tap water. After 1 week, the seedlings were transplanted to pots with sterilized sand (0 to 2 mm grain size, Hornbach, Hannover, Germany) and watered as needed with modified Hoagland solution (Epstein, 1972). After 3 weeks of growth, plants were placed in hydroponics.

The hydroponic experiment was setup as illustrated in figures 5 and 6. In more detail, for each of the studied species: 12 plastic containers with 13L capacity were filled up to a marked water level (~13L) with nutrient solution (table 1) and constantly aerated with small compressors; 8 plants of similar size were weighted together (in order to determine biomass gain per container) and then placed in each container trough round holes in the lid and fixed with soft foam.

The acclimatization to salinity using artificial sea salt (Seequasal GmbH, Münster, Germany) was performed in the first week of hydroponics: on the second day sea salt was added to obtain 10 and on the fifth and sixth days salinity was raised until 15 and 20, respectively. The experiment ran under greenhouse conditions with an average temperature of 22°C (± 1°C). Photoperiod was kept at a 14h/10h light/dark rhythm using sodium vapour lamps (SON-T Agro 400, Philips, Amsterdam, Netherlands) as artificial light source. Water lost by

evapotranspiration and plant consumption was replenished every 2/3 days using only fresh water to ensure the stability of salt and nutrient concentrations.

In this experimental design 4 treatments (4 different nitrogen concentrations) were tested, with 3 replicates (containers) per treatment.

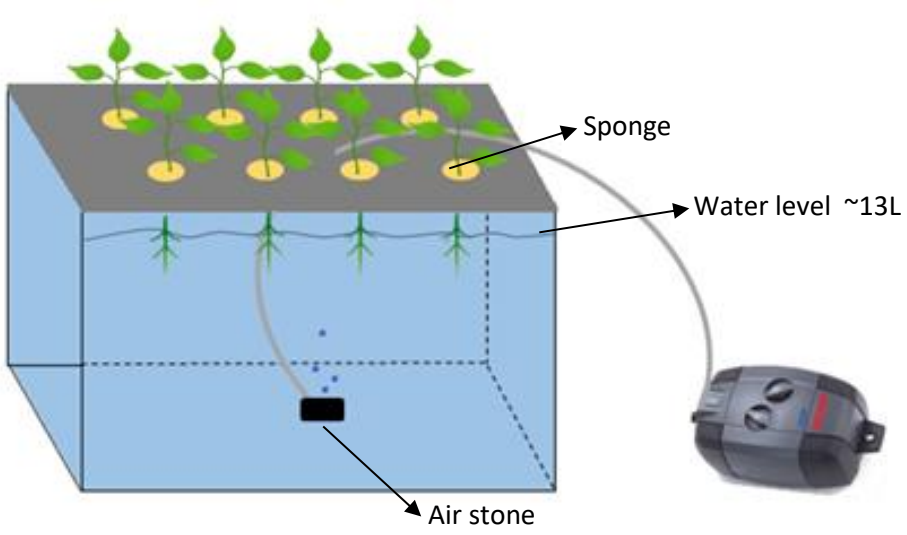

Figure 5. Experimental design for each container.

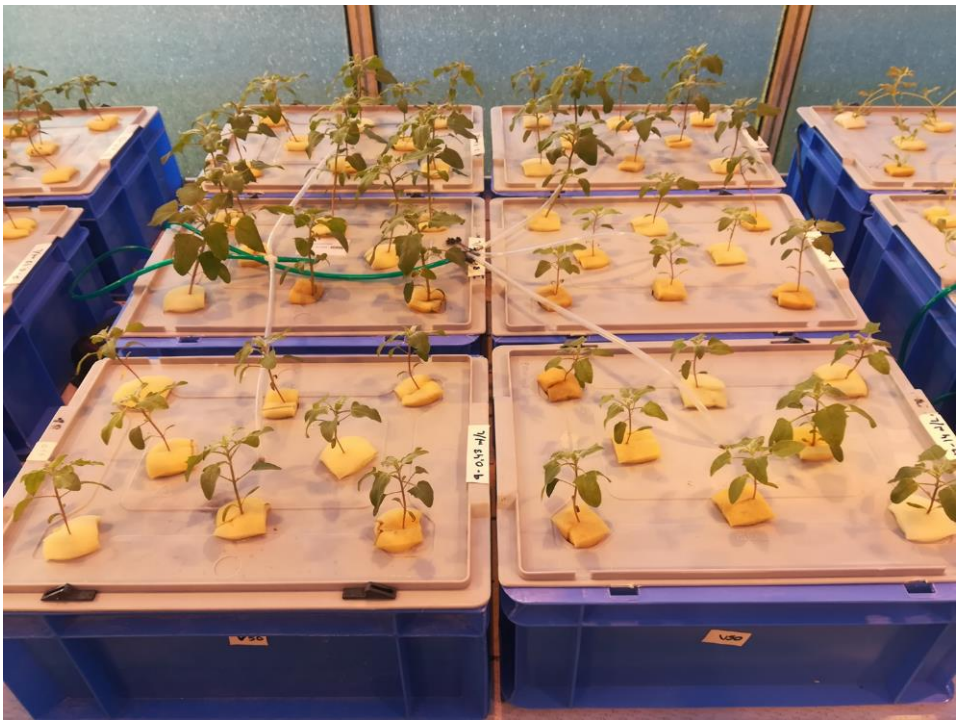

Figure 6. General aspect of the experimental design on the first day of experiment.

As the main objective of the present study was to solely vary the nitrogen concentration, a special modification of the Hoagland solution (Epstein, 1972) was made. Compounds containing nitrate or ammonia (KNO_3, $Ca(NO_3)_2$, $NH_4H_2PO_4$) were removed in order to avoid different sources of nitrogen, but to maintain the presence of the other elements (potassium, calcium and phosphorus) they were replaced by KH_2PO_4 and $CaCl_2$, in the same molarity. As the single source of nitrogen (in order to facilitate calculations and chemical interferences) $NaNO_3$ was used with four different concentrations: 20 mg l^{-1}; 40 mg l^{-1}; 100 mg l^{-1} and 200 mg l^{-1} of nitrogen in the solution, from now on designated as N20, N40, N100 and N200 treatments, respectively.

Table 1. Nutrient solution used in hydroponics experiment.

Compounds	**Nutrient solution concentration**		
	mg l^{-1}	µmol l^{-1}	
Macronutrients			
$NaNO_3$	121,32	1430	**N20**
	242,65	2850	**N40**
	606,83	7140	**N100**
	1189,86	14000	**N200**
KH_2PO_4	272,18	2000	
$CaCl_2$	443,92	4000	
$MgSO_4 *7H_2O$	246,47	1000	
KCl	298,24	4000	
Micronutrients			
KCl	3,73	50	
H_3BO_3	1,55	25	
$MnSO_4*H_2O$	0,34	2	
$ZnSO_4*7H_2O$	0,58	2	
$CuSO_4*5H_2O$	0,12	0,5	
$MoNa_2O_4*2H_2O$	0,12	0,5	
$C_{18}H_{20}N_2O_6Fe$	0,558 Fe		

2.2.Harvest procedure

After 5 weeks of hydroponic conditions for *H. portulacoides* and 4 weeks for *C. quinoa,* the plants were harvested as described:

Chlorophyll content was measured using Minolta SPAD-502Plus leaf chlorophyll meter, in 5 leaves randomly selected by container. At the end of the experiment, all plants from each container were harvested and weighted. Subsamples of *H. portulacoides* leaves and of *C. Quinoa* leaves and tips (as illustrated in fig. 7) from each container were randomly collected (fig.8) and stored at -80 °C.

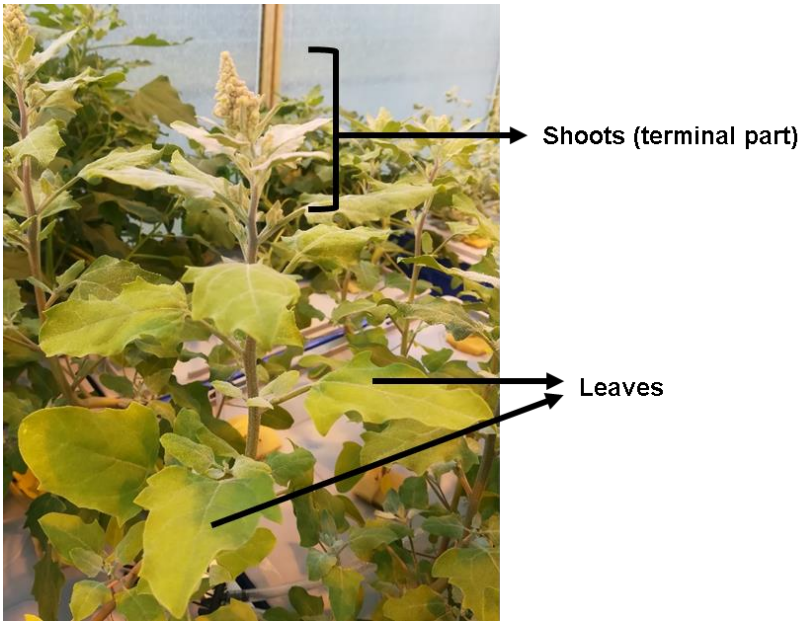

Figure 7. Exemplification of the parts of *C. quinoa* collected for further analysis.

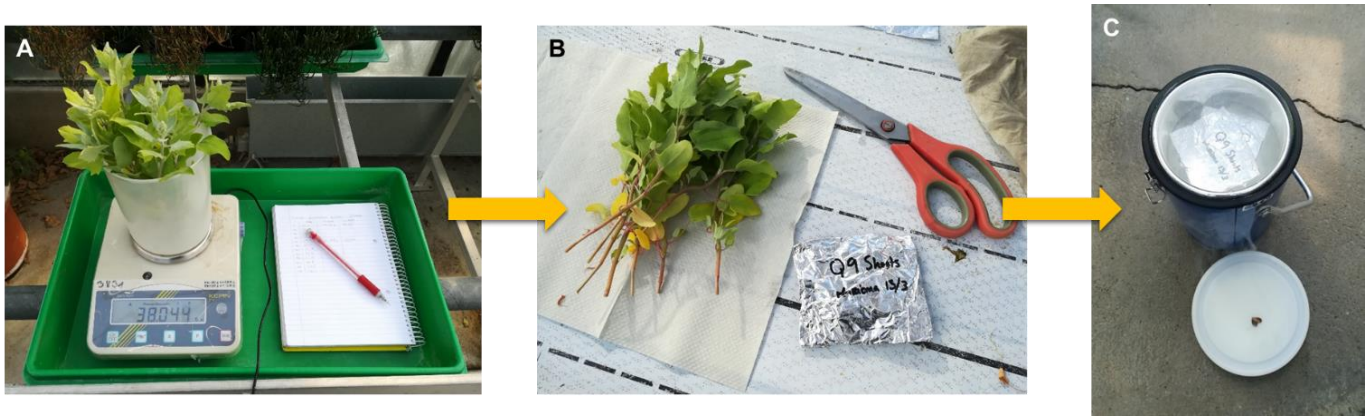

Figure 8. Different stages of the harvest procedure. (A) Weighing of the set of plants from each container; (B) collection of aerial part in aluminium foil and (C) storage in liquid nitrogen.

2.3.Analytical procedure

2.3.1. Characterization of the cultured plants

For all further analysis, frozen plant material was manually grounded in liquid nitrogen to fine powder with a mortar and pestle (fig. 9) and stored at -80°C until analysis.

Figure 9. Grinding of plant material until fine powder.

2.3.1.1. Determination of total phenols, total flavonoids and oxygen radical absorbance capacity (ORAC)

For the determination of secondary metabolites, 50 mg of ground sample was incubated for 10 min in 800 µl of 80% ice-cold methanol with shaking every 2 min. After centrifugation (5 min, 4°C, 13000 rpm) the supernatant was collected. This extraction was performed with

the pellet three times with 400 µl of 80% ice-cold methanol to produce a total of 2 ml of extract. Extracts were used as detailed below.

Total phenols: Following the method by Dudonné, et al., (2009), 100 ml of water was pipetted into each well of a clear 96-wells microplate. Triplicates of 10 µl sample, blank (80% methanol) or Gallic acid standard (5–250 µg ml^{-1}) were added, followed by 10 µl of Folin–Ciocalteu reagent. After incubation for 8 min and addition of 100 µl of 7% Sodium Carbonate, the plate was incubated for 100 min at room temperature and absorbance measured at 765 nm. Total phenols were calculated from a standard curve.

Total flavonoids: Following the method by Dewanto et al., (2002), 150 ml of water was added to each well of a clear 96-wells microplate, then triplicates of 25 ml of sample or catechin hydrate standard (10–400 µg ml^{-1}) or 80% methanol as blank were added followed by 10 µl of 3.75% $NaNO_2$ and an incubation of 6 min. After adding 15 µl of 10% $AlCl_3$ and 5 min incubation, 50 µl of 1M NaOH was added and the total flavonoids content was calculated from a standard curve after absorbance measurement at 510 nm.

ORAC: The determination of the oxygen radical absorbance capacity followed the method by Huang, et al., (2002) and the method by Gillespie, et al., (2007). Samples were diluted (1:40) in phosphate buffer (75 mM, pH 7.4). A black 96-wells microplate was kept on ice, where 20 µl of standards (6.25-50 µM Trolox in phosphate buffer), sample or blanks (phosphate buffer) were pipetted followed by 120 µl of fluorescein (1:10000 diluted in phosphate buffer from 1.12 mM stock solution). The plate was incubated for 15 min at 37°C and the fluorescence was measured at time point 0 at 485/520 nm. Then 80 µl of freshly prepared 62mM 2,2'-azobis(2-amidino-propane) dihydrochloride were added into each well and the fluorescence at 485/520 nm was measured every minute for 80 min. The antioxidant capacity was quantified using a standard curve.

2.3.1.2. Determination of ascorbic acid content

The determination of AA, dehydroascorbic acid (DHA) and total ascorbic acid (TAA) followed well established protocols (Gillespie and Ainsworth, 2007; Kampfenkel et al., 1995; Stevens et al., 2006; Ueda et al., 2013). Frozen ground plant material (50 mg) was shaken with 500 µl of ice-cold MPA (6 %) and suspended on ice for 15 min until centrifugation (20 min, 4°C, 14000 rpm) and stored on ice before use. Ten microliters of cold phosphate buffer (75 mM, pH 7.0) and 20 µl of blank, standard (1–0.0625mM ascorbic acid in 6% MPA) or sample were added to a clear 96-wells microplate**.** For reduction of DHA, 10 µl of 10 mM dithiothreitol (DTT) were added to every second sample for TAA determination. After a quick shake at 900rpm and 15 min incubation, 10 µl of 0.5% N-ethylmaleimide (NEM, solved in 70% ethanol) were added to the same second sample and 20 µl of water were added into the other wells to compensate the volume of DTT and NEM. After 2 min incubation, 150 µl of reaction mixture (five parts of 10% TCA, two parts of 3% $FeCl_3$, four parts of 43% H_3PO_4 and four parts of 4% a-a'-bipyridyl solved in 70% ethanol) was added. The plate was incubated for 60 min at 37°C (shaken every 10 min) and the absorption at 525 nm was measured. The difference between the measured TAA and AA is the calculated DHA.

All of the previously described spectrophotometric measurements were performed in an ELISA SYNERGY MX microplate reader (BioTek, Bad Friedrichshall, Germany).

2.3.1.3. Determination of Carbon and Nitrogen (C, N) content

Approximately 1 g of plant biomass was placed in the oven for 48h at 60°C to remove water content. Aliquots of dried biomass (10-15 mg) were weighted and foiled in aluminium boats, which were then pressed to minimize oxygen content and analysed for C and N content through a C-N-S Elemental Analyzer (Vario EL III Elementar Analyzer, Elementar Analysensysteme GmbH, Langenselbold, Germany). A High Organic Sediment IVA33802150

standard (IVA Analysentechnik, Germany) was used as reference material. The percentage of carbon and nitrogen in each sample was obtained through combustion of the sample (total C was quantified as CO_2 and total N was quantified as N_2).

2.3.1.4. Elemental Analysis

Following the method by Weese et al., (2015), about 38 mg of previously dried samples were placed in scintillation glasses and incinerated in a muffle furnace for a minimum of 8h at 480 °C. After cooling at room temperature, an extraction with nitric acid was performed and the extracts were filtered. The extract was analysed for the 24 elements mentioned below by inductively coupled plasma optical emission spectrometry (ICP-OES) (iCAP 6000 ICP Spectrometer, Thermo Fisher Scientific Corporation, Waltham, Massachusetts, USA). As reference, a Roth multi element standard (24 elements: Al (1000), As (100), Ba (500), B (500), Ca (1000), Cd (100), Co (500), Cr (500), Cu (500), Fe (1000), Hg (10), K (1000), Li (100), Mg (1000), Mn (500), Na (1000), Ni (500), Pb (500), Rb (500), Sr (500), Te (500), Ti (500), V (500), Zn (500), 100 µg ml^{-1}, Carl Roth) and ICP single element standards for potassium (K), calcium (Ca), magnesium (Mg), sodium (Na), sulfur (S), phosphorus (P) and iron (Fe) (10000 µg ml^{-1}, CPI International).

2.3.2. Characterization of the culture medium

A sample of the culture medium from each container was collected on the first and last days (harvest day) to characterize the hydroponic conditions. Samples were collected using 20 ml plastic bottles and stored at -20 °C until nutrient analysis.

2.3.2.1. Determination of nutrients concentration

Nitrate: an adaptation of previously published protocols (Velghe and Claeys, 1985; Zhang and Fischer, 2006) was followed. Ten µl of 10% sulfamic acid were pipetted into each well of a clear 96-well microplate, followed by 80 µl of sample (with a dilution of 1:60), blank or standard (1-10 mg l^{-1}, salinity of 15). After a 30 min water bath (80°C) and a quick centrifugation (1200rpm), 10 µl of 2.5% resorcinol and 150 µl of concentrated sulphuric acid were added and the plate was placed in a water bath at 80°C for 120 min. After cooling down in the dark at room temperature for 15 min and a quick centrifugation (1200 rpm) the absorption was measured at 505 nm.

Phosphate: Based on the method of Hernandez-Lopez & Vargas-Albores (2003) and Murphy & Riley (1962), 30 µl of reaction solution (0.6% ammonium heptamolybdate, 12.75% sulphuric acid, 1.08% ascorbic acid, and 0.0163% each of antimony and potassium tartrate) were added to each well followed by 250µl of sample, blank or standard (0.1-2.8 mg l^{-1}). After a 10 min incubation at room temperature and a quick centrifugation (1200 rpm) absorbance was measured at 655 nm.

2.4.Statistical analysis

All the treatments were carried out in triplicates: three containers per treatment with 8 plants each.

Culture medium samples from each container of both plants were tested in triplicate (technical replicates) for the determination of the concentration of nutrients (phosphate and nitrate) and mass balance calculations. Significant differences between treatments in terms of nitrogen (in the form of nitrate) and phosphorus (in the form of phosphate) removal were tested using One Way analysis of variance (ANOVA) and Holm-Sidak post-hoc analysis.

Plant biomass from each container was used in triplicate (technical replicates) for the determination of total flavonoids, total phenols and ORAC, and in duplicate for ascorbate (due to protocol constraints, since the layout of samples in the 96-well microplate allowed the use of only 2 replicates per sample.). *Halimione portulacoides* data was tested for significant differences between treatments in terms of biomass gain, chlorophyll content, total flavonoids, total phenols, ORAC, ascorbate and nitrogen content using One Way ANOVA as well. A Two Way ANOVA and Holm-Sidak post-hoc analysis were performed with *C. quinoa* data to test significant differences for the same parameters (biomass gain, chlorophyll, antioxidants and nitrogen content) between treatments and between shoots and leaves of each treatment.

Statistical analyses were performed with SigmaPlot software (version 12.3). For all tests a significance level of 0.05 was used.

Principal components analysis was performed for each plant species using PRIMER software (version 6.1.13) to seek the contribution of each variable and explain the variance in order to visualize the proximity and relation between variables.

3. Results

3.1. Plant characterization

During the experiment visual differences in the plants were noticeable, particularly in size and colour.

For *H. portulacoides*, visual differences in size were observed, especially in the N20 treatment. Under these conditions plants were less developed displaying lower biomass (fig. 10).

Figure 10. *H. portulacoides* examples of each nitrogen treatment on harvest day. N20, N40, N100 and N200 from left to right, respectively.

After harvesting and analysing the plant material of *H. portulacoides*, the plants in the two highest nitrogen treatments (N100 and N200) presented significantly higher biomass gain and chlorophyll content comparatively to the lowest treatment (fig. 11). The treatment where the plants grew more, on average, was N100, and the highest biomass gain variability was observed in N200 treatment.

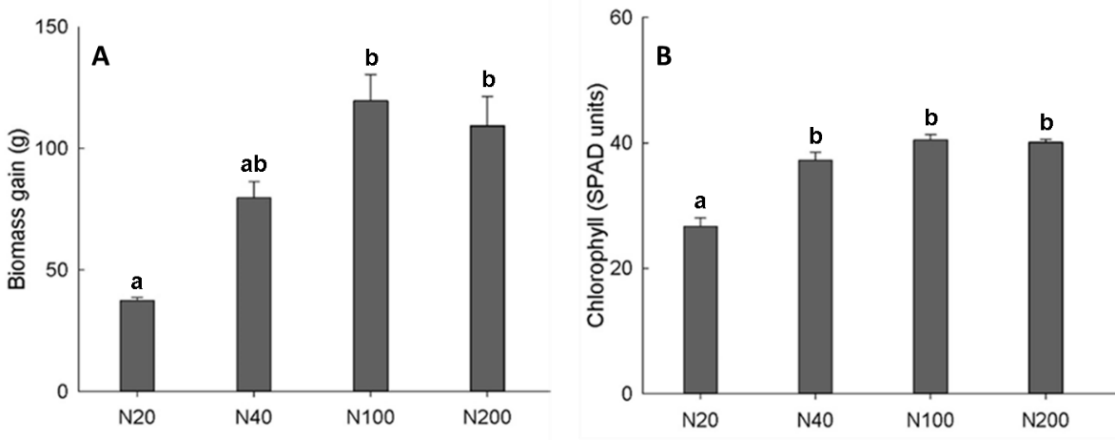

Figure 11. Biomass gain in grams per container (A) and leaf chlorophyll content on harvest day (B) for *H. portulacoides* in each nitrogen treatment. Bars represent mean and standard error of three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments.

Visual differences were most obvious for *C. quinoa*, since the plants in the lowest nitrogen treatment (N20) were much smaller and presented a yellowish colour (particularly in the leaves) when compared with the other treatments (fig. 12).

Figure 12. *C. quinoa* examples of each nitrogen treatment on harvest day. N20, N40, N100 and N200 from left to right.

Figure 13A represents the biomass gain in grams per container between the beginning and end of the experiment. Based on statistical results, the plants in the N100 and N200

treatments grew significantly more when compared with N20 and N40. In terms of chlorophyll content it's evident the difference between N20 and other treatments (fig. 13B) which is in accordance with the visual differences in leaf colour.

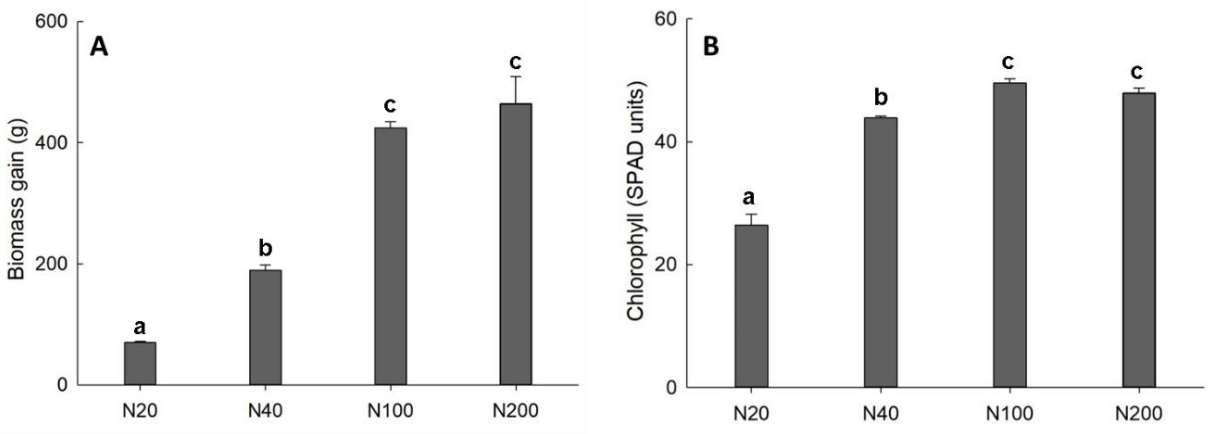

Figure 13. Biomass gain in grams per container (A) and leaf chlorophyll content on harvest day (B) for *C. quinoa* in each nitrogen treatment. Bars represent mean and standard error of three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments.

3.1.1. Antioxidant content

Total flavonoids, total phenols, ascorbic acid content and ORAC value obtained in *H. portulacoides* are represented in fig. 14. Although N20 and N100 treatments presented higher flavonoid content (on average) when compared to other treatments, the differences were not significant ($p=0.271$) between treatments. A similar result was obtained for ascorbic acid, where the differences between treatments are slight.

However there were significant differences ($p<0.001$) between treatments in terms of phenols content and ORAC value, where, for both tests, the N20 and N40 treatments presented significantly higher antioxidant content and capacity than N100 and N200. Treatment N20 showed statistically higher phenols content and ORAC value when compared with remaining treatments.

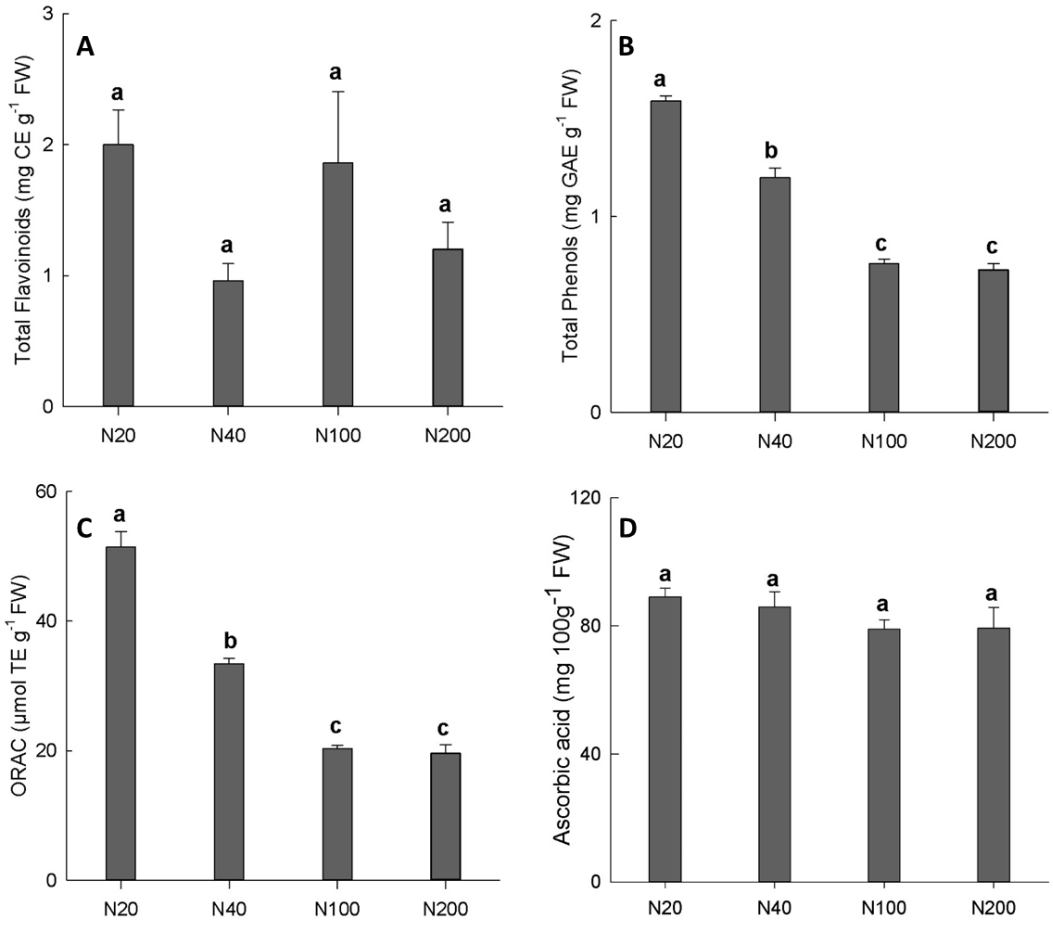

Figure 14. Antioxidant content of *H. portulacoides* after 5 weeks of hydroponics. Total flavonoids (A), total phenols (B), ORAC value (C) and ascorbic acid (D) content are represented in the different nitrogen treatments. Bars represent mean and standard error of three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments. CE: catechin equivalents; GAE: gallic acid equivalents; TE: trolox equivalents.

Antioxidant content analyses of *C. quinoa* (fig. 15) showed overall higher concentrations in N20 treatment when compared to the other treatments.

Also noticeable for all of the antioxidant tests was that leaves always presented higher content in the two lowest nitrogen treatments when compared to the shoots, whereas in the two highest treatments, the shoots presented a higher antioxidant concentration compared to the leaves.

Total flavonoid content was higher in N20 treatment, particularly in the leaves, while the remaining treatments presented similar values.

The N20 treatment presented higher phenol concentration comparatively to the highest treatments, especially in the leaves. There were no significant differences between shoots of different treatments but the leaves of N20 and N40 showed significantly different concentrations. There were also differences among plant parts (shoots and leaves) of N20 and N100 treatments.

ORAC values were significantly different in the leaves of N20 treatment when compared to the leaves of N100 and N200. There were no significant differences between shoots of different treatments and among plant parts of the same treatment.

Shoots and leaves of the same treatment presented significant differences in all of the experimental treatments in terms of ascorbic acid content. A significant difference was visible between the lowest (N20 and N40) and the highest (N100 and N200) treatments.

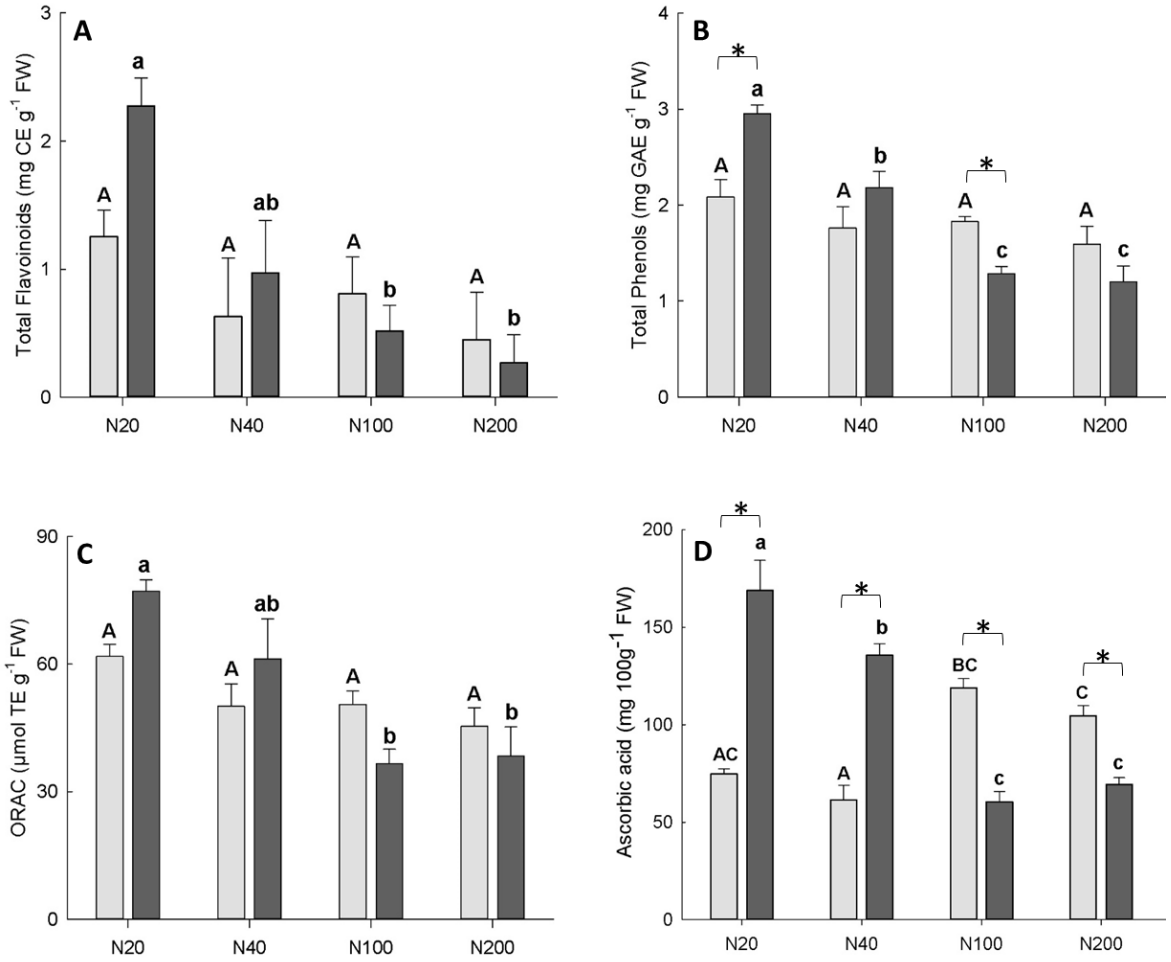

Figure 15. Antioxidant content of shoots (light grey) and leaves (dark grey) of *C. quinoa* after 4 weeks of hydroponics. Total flavonoids (A), total phenols (B), ORAC value (C) and ascorbic acid (D) content are represented in the different nitrogen treatments. Bars represent mean and standard error of three replicates. Different capital letters indicate significant differences ($p<0.05$) between shoots of each treatment and different lowercase letters indicate significant differences ($p<0.05$) between leaves of each treatment. Asterisks represent significant differences ($p<0.05$) between shoots and leaves of the same treatment. CE: catechin equivalents; GAE: gallic acid equivalents; TE: trolox equivalents.

3.1.2. Elemental content

Figure 16 represents the nitrogen and carbon content in *H. portulacoides* after the experiment. An increase in nitrogen content in the plant with increasing nitrogen in the nutrient solution was observed, with significant differences between N20 and N40 treatments but no significant differences between N100 and N200 treatments. The highest percentage was achieved in N200 treatment with an average of 5% nitrogen content in

plant material. The carbon content in the different treatments presented similar values, with all the samples containing between 30% and 33% of carbon.

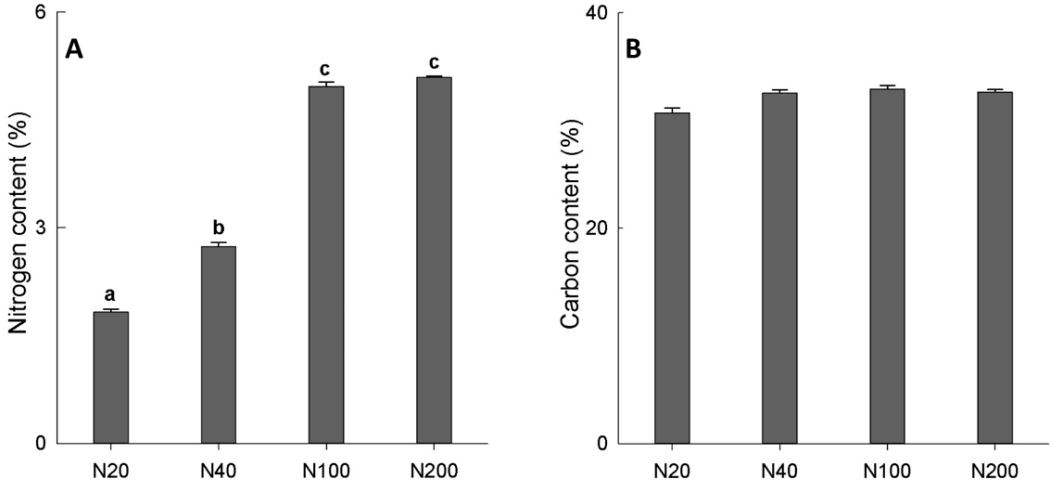

Figure 16. Percentage of nitrogen (A) and carbon (B) in *H. portulacoides*. Bars represent mean and standard error of three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments.

In terms of nitrogen content of *C. quinoa* (fig. 17A), statistical differences ($p<0.05$) were noticed between the four nitrogen treatments and between all the shoots and leaves in the same treatment. With increasing nitrogen availability in the nutrient solution there was an increase in nitrogen content in the plants, with the shoots holding the highest percentages. Regarding the carbon content (fig. 17B) no differences were observed between treatments and plant parts. The average carbon content was approximately 36%.

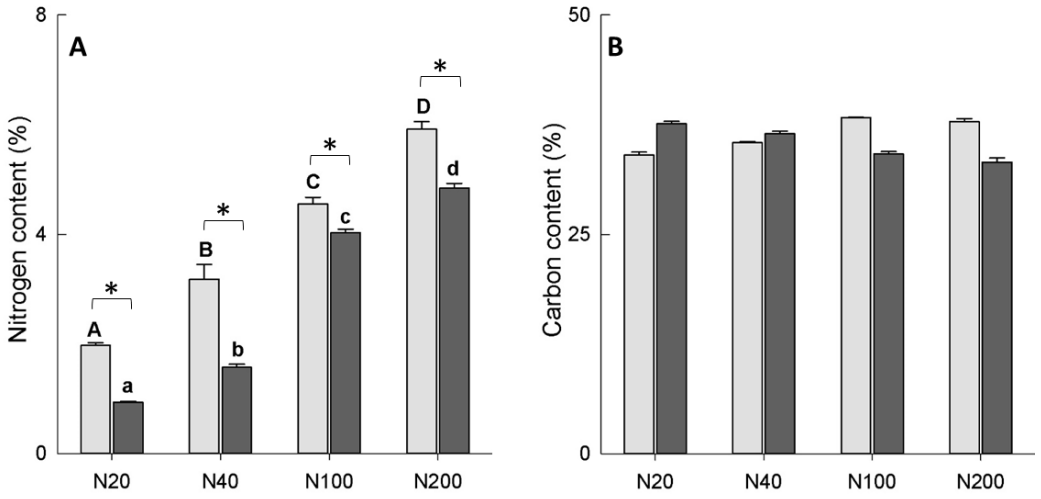

Figure 17. Percentage of nitrogen (A) and carbon (B) content in the shoots (light grey) and leaves (dark grey) of *C. quinoa.* Bars represent mean and standard error of three replicates. Different capital letter indicate significant differences ($p<0.05$) between shoots of each treatment and different lowercase letters indicate significant differences ($p<0.05$) between leaves of each treatment. Asterisks represent significant differences ($p<0.05$) between shoots and leaves of the same treatment.

Figure 18 represents the element content of *H. portulacoides* in the different treatments. The prevailing element for all treatments was sodium, which is comprehensible since the experiment was carried out under saline conditions (20). Element content was similar throughout the nitrogen treatments with exception of potassium, which was slightly higher in N20 treatment. Magnesium, iron and zinc were present in trace amount.

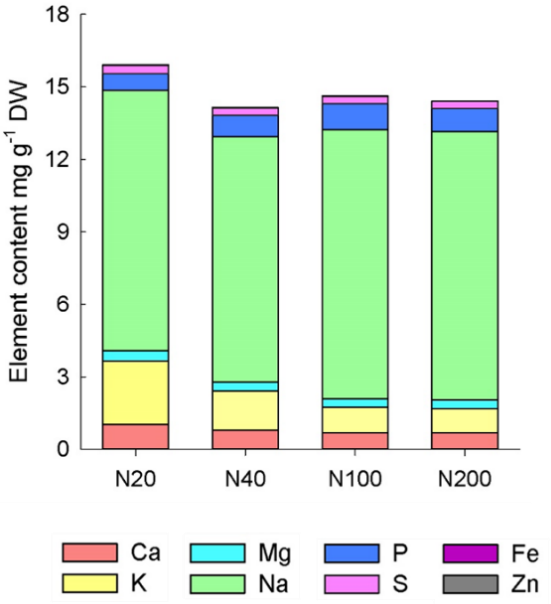

Figure 18. Element content of *H. portulacoides* after 5 weeks of hydroponics.

Regarding the element content of the shoots and leaves of *C. quinoa*, fig. 19 shows that, once more, the predominant element was sodium and magnesium, iron and zinc were found in trace amount. In the leaves (fig. 19 right) differences were clearly visible between the two lowest and two highest nitrogen treatments. The leaves of N100 and N200 treatments had higher content of sodium and calcium compared to N20 and N40.

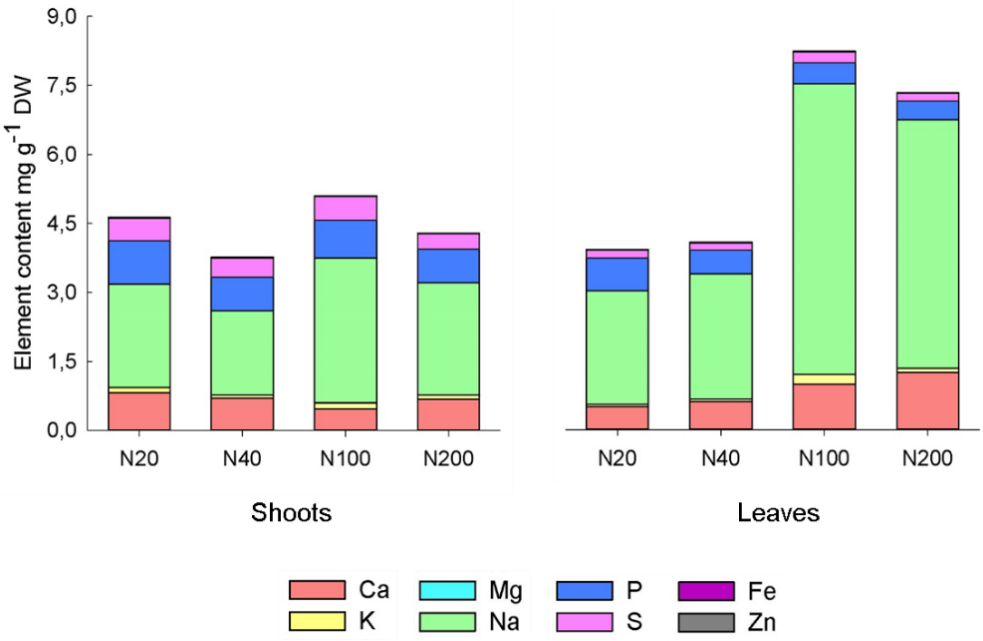

Figure 19. Element content of shoots (left) and leaves (right) of *C. quinoa* after 4 weeks of hydroponics.

3.1.3. Principal Components Analysis

Principal components analysis of *H. portulacoides* data is displayed in fig. 20 and it's visible a separation between nitrogen content and calcium, ORAC, phenols and potassium which moved along opposite sides of PC1 axis. A similar separation is notorious among groups of treatments, which also distributed themselves in opposite sides of PC1, showing a correlation between the samples of N20 treatment and higher antioxidants, calcium and potassium content and, contrastingly, a correlation between higher nitrogen treatments and higher nitrogen content in the plants. Both axis presented a cumulative explained variation of 73.4%.

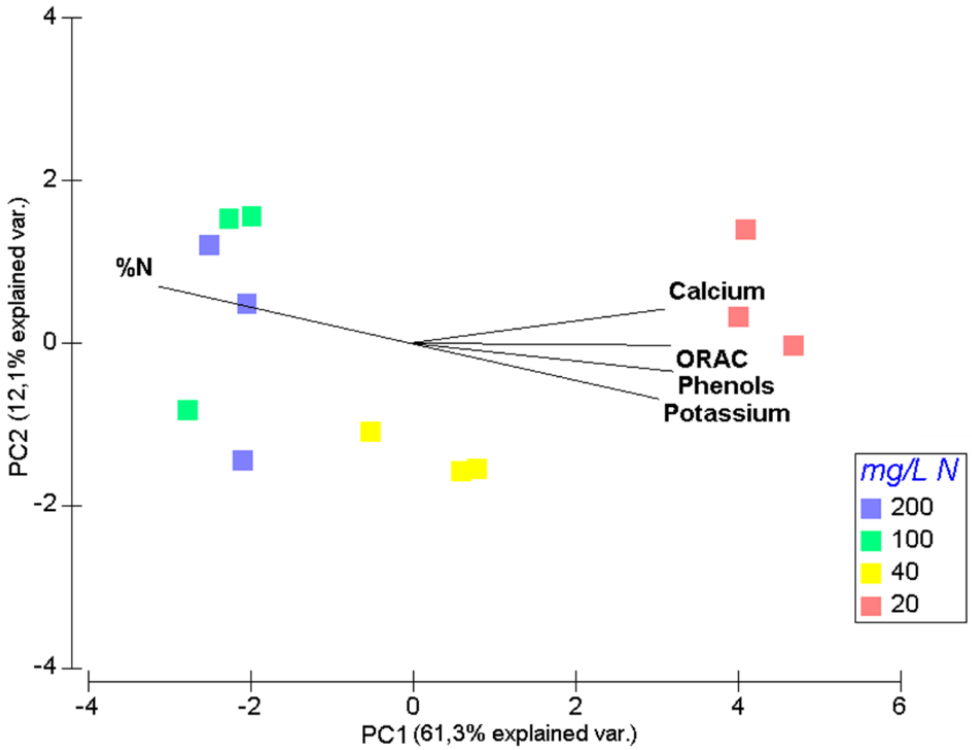

Figure 20. Results of principal components analysis for total flavonoids, total phenols, ascorbate, ORAC, calcium, potassium, magnesium, sodium, phosphorus, sulphur, iron, zinc and nitrogen content of *H. portulacoides*. Only variables with a Pearson correlation superior to 0.9 are displayed. Different treatments are indicated by different colours.

Figures 21A and B show principal components distribution for *C. quinoa* data. There was not a clear separation between shoots of different treatments (fig. 21A), however, the samples of higher nitrogen treatments presented an association with higher nitrogen content since they were distributed towards the positive side of PC1. Leaves of *C. quinoa*, on the other hand, presented a clear separation along PC1 axis, which explained 68.3% of variation between groups. The lowest nitrogen treatments (N20 and N40) distributed along the negative side of PC1 presenting a correlation with higher phenols and ascorbate content and, contrastingly, the leaves of the higher nitrogen treatments moved along the positive side of this axis, correlating with higher nitrogen and elemental content. This segregation of leaf samples is accompanied by photos of *C. quinoa* in N20 treatment (fig. 21C) and N200 treatment (fig. 21D) where the visual differences in leaf size and colour are very clear.

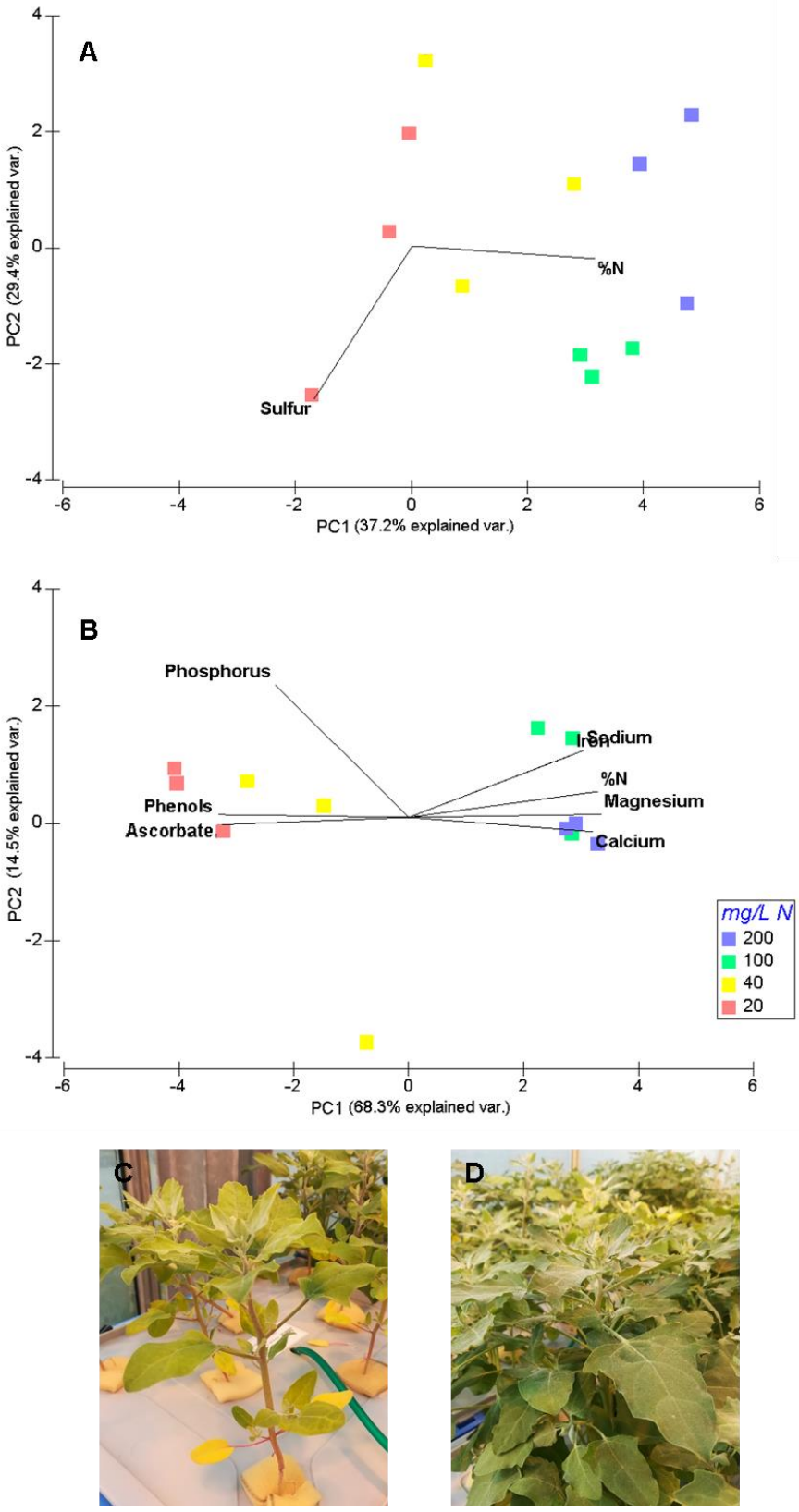

Figure 21. Results of principal components analysis for total flavonoids, total phenols, ascorbate, ORAC, calcium, potassium, magnesium, sodium, phosphorus, sulphur, iron, zinc and nitrogen content of *C. quinoa* shoots (A) and leaves (B); exemplars of 3rd week of hydroponics in N20 (C) and N200 (D) treatments. Only variables with a Pearson correlation superior to 0.9 are displayed. Different treatments are indicated by different colours.

3.2.Nutrients concentration

Nitrate and phosphate concentration in the nutrient solution was determined in the beginning and end of the experiment to verify the extraction capacity of the plants. Figure 22 shows those results for *H. portulacoides* where it's visible that there was a higher removal of nitrogen (in the form of nitrate) in N40 (63%) and N100 (55%) when compared to the other treatments but in concentration values the treatments where the most nitrogen (in the form of nitrate) was significantly removed were N100 and N200. For phosphorus (in the form of phosphate), N100 and N200 are the treatments with the higher removal percentages and concentrations.

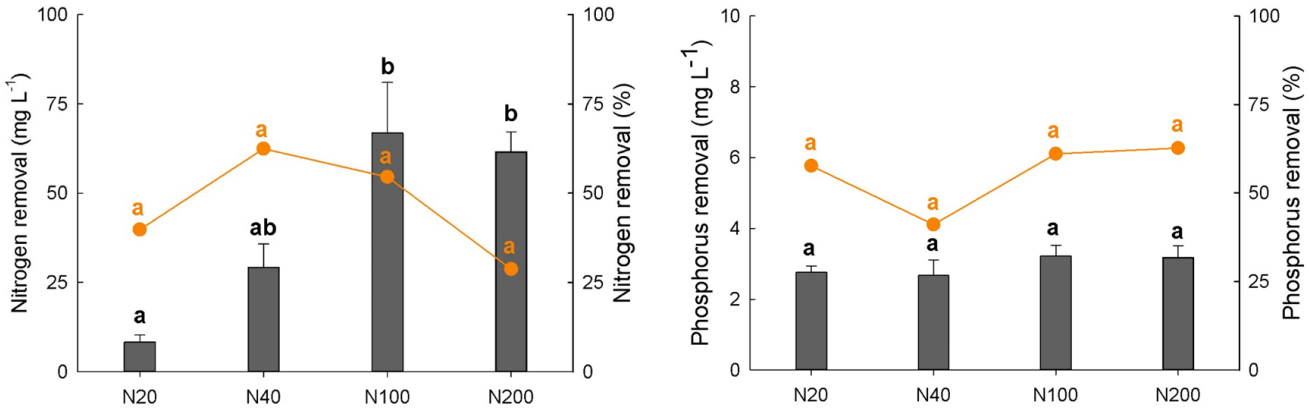

Figure 22. Nitrogen (in the form of nitrate) (A) and phosphorus (in the form of phosphate) (B) removal in the experiment with *H. portulacoides*. Grey bars represent mean and standard error of the removed nutrient concentration of three replicates and orange line represents mean percentage values of removed nutrient in three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments.

For *C. quinoa* the results of nitrogen (in the form of nitrate) and phosphorus (in the form of phosphate) removal from the nutrient solution are represented in fig. 23. The removal percentage of nitrogen in the N100 and N200 treatments was significantly higher ($p<0.05$) than in N20. The N100 treatment had the highest removal with an average of 85% nitrogen removed whereas the N200 treatment had the highest removal concentration of 700 mg L^{-}

[1] (which corresponds to approximately 79%) of nitrogen removed after 4 weeks of hydroponics. In terms of phosphorus removal there were no significant differences between treatments, with the highest phosphorus removal percentage present in the N200 treatment (approximately 90%).

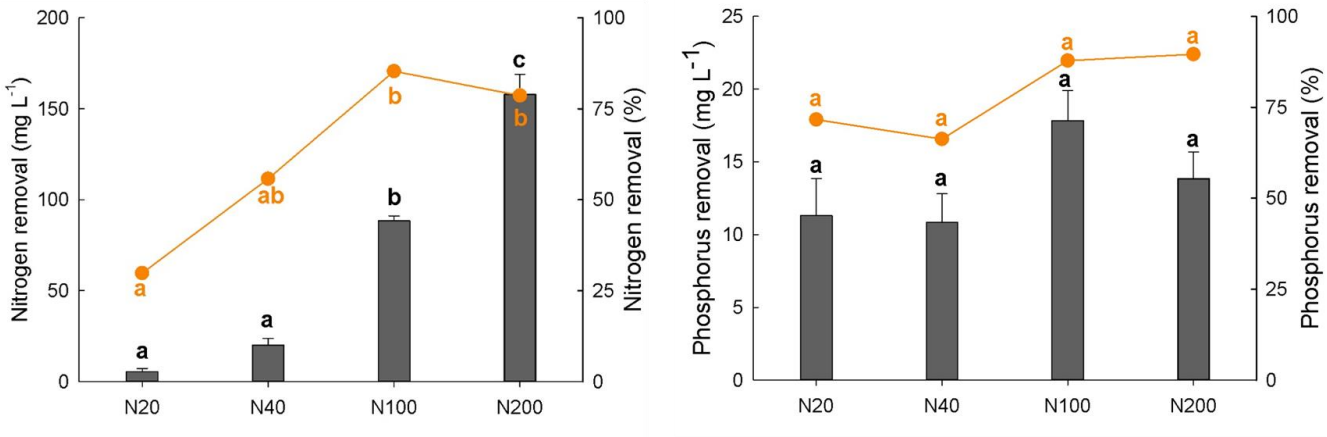

Figure 23. Nitrogen (in the form of nitrate) (A) and phosphorus (in the form of phosphate) (B) removal in the experiment with *C. quinoa*. Grey bars represent mean and standard error of the removed nutrient concentration of three replicates and orange line represents mean percentage values of removed nutrient in three replicates. Different lowercase letters indicate significant differences ($p<0.05$) between treatments.

4. Discussion

Nitrogen is an essential nutrient for plant health since it serves as the basis for proteins, vital molecules for the growth, development and enzymatic activity of the plant (Silva and Uchida, 2000). It is also important for the chlorophyll molecule and thus, for photosynthesis (Silva and Uchida, 2000). Therefore, the deficit of this nutrient in the soil or in the plant tissue is associated with poor growth and chlorosis, a condition characterized by a pale green to light yellow colour in the older leaves, caused by the translocation of nitrogen from older to younger tissues (Silva and Uchida, 2000). In some crops can also cause early maturation, resulting in loss of yield and quality (Silva and Uchida, 2000). Due to this fact, this study showed that both plant species in the N20 treatment didn't present a successful biomass gain and chlorophyll content when compared with the remaining treatments, suggesting that the lowest concentration of nitrogen used in the experience was at limiting levels, not sufficient for the maintenance of normal plant health. It is also known that nitrogen deficiency stimulates root development, in order to intensify uptake of nutrients from a nutrient limited environment, and might inhibit shoot growth (Kováčik and Bačkor, 2007).

When combining growth and chlorophyll content data with antioxidant analysis, it becomes clear that plants in N20 treatment were under stress conditions. In *H. portulacoides* total flavonoids and ascorbic acid content results didn't allow to the take many conclusions about the antioxidant activity of the plant, whereas, total phenols and ORAC analysis revealed that the plants with low nitrogen availability showed higher antioxidant production, particularly in N20 treatment. Elevated antioxidant production has been associated with stressful conditions. *Halimione portulacoides* has previously shown high ORAC values as a short term response to salinity stress (Boestfleisch et al., 2014; Boestfleisch and Papenbrock, 2017). Bazzaz et al. (1987) also suggested that a high antioxidant production might imply a compromise in growth as form of resource allocation, which might explain the lower biomass yield in plants with high antioxidant content. However, if the stress conditions were to be applied shortly before harvest, perhaps an

increase in antioxidant content would be observed with no negative impact on biomass. Some studies suggest that pre-harvest abiotic stress induction (Rosalie et al., 2015) or pre-harvest application of algal extracts (Fan et al., 2011) can increase antioxidant content, hence, improving crop nutritional and commercial value since these compounds have several pharmacological applications (Buhmann and Papenbrock, 2013b; Ksouri et al., 2012)

As for *C. quinoa* antioxidant production, lower nitrogen availability induced a higher antioxidant production in the leaves rather than in the shoots and these values decreased with increasing nitrogen concentration in the hydroponic solution. Similarly to *H. portulacoides*, these observations reveal that the concentration that triggers the stress conditions is between 40 mg L^{-1} and 20 mg L^{-1}.

A previous experiment with apple tree showed that reduced availability of nitrogen and potassium was followed by an accumulation in the leaves of phenylalanine ammonia-lyase (PAL, a precursor of phenylpropanoid metabolism) (Tan, 1980). This enhanced PAL activity might contribute to the increase of several phenolic compounds, such as flavonols, which were found in increasing concentrations of tomato plant leaves in response to nitrogen stress (Stewart et al., 2001).

The results of this study showed a higher variability in plant nitrogen content between treatments than plant carbon content. As expected, plants that grew in the hydroponic solution with higher nitrogen availability tend to have higher internal nitrogen concentrations, as the results show, which then is reflected in improved plant biomass (Pilbeam, 2018).

An adequate nitrogen supply potentiates an efficient photosynthetic process with optimal CO_2 fixation and, consequentially, a proper growth and development of the plant (Nunes-Nesi et al., 2010). This fixed carbon is also required for inorganic nitrogen assimilation since it provides the C-skeletons that act as nitrogen acceptors (Nunes-Nesi et al., 2010). *Halimione portulacoides* showed a higher carbon content along with higher chlorophyll content in the higher nitrogen treatments. This is also in accordance with the higher phenol content in the lowest treatments since, under nutrient stress conditions, carbon becomes

a “cheaper” nutrient to obtain leading to the increased production of carbon based antioxidants like phenols (Bryant et al., 1983).

The situation becomes more complex for *C. quinoa* and the previous argument might not be applicable to this species since it presented similar results for chlorophyll and antioxidant production as *H. portulacoides* but not for carbon content. The leaves of *C. quinoa* presented a decreasing carbon content as the nitrogen concentration in the water increased, whereas the shoots presented the opposite pattern. This pattern is particularly noticeable in N100 and N200, leading to the possibility that, perhaps the carbon, in the treatments with higher nitrogen availability, is being mobilized from the leaves to the shoots where antioxidant production is slightly higher. This decreasing concentration of carbon in the leaves seems to have no negative effect in the photosynthesis, indicating proper assimilation of CO_2. So, these results might be explained by resource allocation during reproductive development, where the carbon necessary in photosynthetic tissues is mobilized to reproductive structures (Bazzaz et al., 1987), the shoots, although, in this case, no adverse effect in photosynthetic capacity is noticed.

Halophytes can present different antioxidant content under different salinity concentrations (Slama et al., 2017). Halophyte *Sesuvium portulacastrum* L. presented a polyphenol content of 6.67 mg GAE g^{-1} DW under 400 mM NaCl (Slama et al., 2017). *Salicornia persica* and *Sarcocornia fruticosa* presented increasing polyphenol content with increasing salinity in hydroponic solution, ranging from 1.05 to 2.05 mg GAE g^{-1} FW of total polyphenols in 50% and 100% seawater, respectively (Ventura et al., 2011). Similar values were obtained in this study, particularly *C. quinoa*, which presented the highest total phenols content. But there are halophyte species with higher phenols content. Shoots of *Suaeda fruticosa* Forssk collected in the wild presented a polyphenol content of 37.1 mg GAE g^{-1} DW and flavonoid content of 26.2 mg CE g^{-1} DW (Oueslati et al., 2012).
Nevertheless, it is also possible to compare the nutritional value of halophytes to some of freshwater species.
Nutritionally, the higher antioxidant capacity (ORAC) values obtained in this study for both plant species are comparable to that of some red fruits (Wu et al., 2004). As for total

phenols content, the plants with lower nitrogen availability presented values similar to that of spinach, broccoli or asparagus (Wu et al., 2004) and plants with higher nitrogen availability showed phenolic values comparable to carrots, some beans or tomato (Kevers et al., 2007; Wu et al., 2004). Thus, showing that the consumption of *H. portulacoides* and *C. quinoa* produced in the experimental conditions of the present study, can be as rich in antioxidants as common foods from traditional agriculture.

When compared with other cereals, like rice and corn from traditional agriculture, *C. quinoa* shows a higher mineral content, particularly in calcium (87mg $100g^{-1}$ DM), potassium (907mg $100g^{-1}$ DM), iron (9.47mg $100g^{-1}$ DM) and magnesium (362mg $100g^{-1}$ DM), as reviewed by Nowak et al. in 2016. In this study, the obtained values for the content of these minerals in this species were lower, although it's not possible to make a direct comparison since production methods are different. Also, elemental analysis for *H. portulacoides* showed lower concentrations when compared with specimens collected in the wild (Neves et al., 2007). Perhaps, these results are an expression of the fact that, in this study, the hydroponic solution was never replenished, only the water evaporation loss was compensated, so, as the nutrients were absorbed, plants were closer to a nutrient limited state overtime, which might have affected mineral content at the end of the experiment. Whereas in the natural environment, plants are adapted to a balanced flow of nutrients resulting from a complex ecological system (Townend et al., 2011).

This study also assessed the capacity of these halophytes to efficiently extract nutrients from the hydroponic solution. Both species revealed promising results with *C. quinoa* particularly standing out with 85% and 89% nitrogen (in the form of nitrate) and phosphorus (in the form of phosphate) removal, respectively, while *H. portulacoides* reached 62% nitrogen and phosphorus removal. These nutrient removal values are presented as a percentage of removal relatively to the initial nutrient concentration in the solution.

Thus, for *H. portulacoides*, the treatment with the highest nitrogen removal percentage is N40 (62%) but, in terms of nutrient concentration the treatment where the plants absorbed

more nitrogen was actually N100 (approximately 300 mg l^{-1} nitrogen removed). A similar result was obtained for *C. quinoa* with a nitrogen removal of 85% in N100 treatment but the maximum nitrogen absorbed was approximately 700 mg l^{-1} in N200 treatment.

Possibly, *H. portulacoides* in the N100 and N200 treatments reached its absorption limit of nitrogen since the concentration of nutrient removed didn't differ much between these two treatments (although a kinetics study with at least one more concentration above N200 would be required to confirm the plateaux). But, when calculating this removal values as a percentage relatively to initial concentration it translates into a relatively lower value, when in fact, particularly *C. quinoa* results, showed that with higher nitrogen availability, a bigger concentration of nitrogen is consumed. The latter species presented, under the studied experimental conditions, the greater tolerance to elevated concentrations of nitrogen.

This study indicates that both plant species present potential to be integrated in saltwater facilities as bioremediation agents and, especially *C. quinoa*, seems to tolerate and present a better performance even with high nitrogen availability.

The integrated production of plants and aquaculture becomes efficient since it has the potential to minimize the environmental impacts of the effluents and to increase the economic return, by offering product diversity and improving resource use efficiency with a circular production (Boxman et al., 2017; Granada et al., 2016).

Some studies show that hydroponic culture can produce crop vegetables and fruits with equal or better yields and antioxidant composition as soil culture (Buchanan and Omaye, 2013; Chandra et al., 2014; Sgherri et al., 2010). Additionally, working with hydroponic culture allows a complete control of nutrient supply (Sgherri et al., 2010), a more hygienic harvest, with no weeds and soil pests (Buhmann et al., 2015) and, when performed under greenhouse conditions eliminates the risk of weather fluctuations and allows for fine-tuned environmental conditions (Chandra et al., 2014).

The results of this experiment showed that in N40 treatment both species presented, overall, good health status and, in the treatments above no significant differences in

antioxidant content and biomass gain were observed. This might come as an advantage, since it suggests that both species might tolerate the natural fluctuations in the concentration of an aquaculture effluent's constituents (depending on type of culture system, rate of production, type of fish feed and feed conversion ratio) (Piedrahita, 2003), without risking production quality.

Both species already have commercial applications and established market values. *Chenopodium quinoa* is already established in the international market and is mainly produced in traditional agriculture (Saleh et al., 2010). This plant also has a broad variety of uses: the leaves and the grains can be consumed raw or cooked and can be ground into flour (Montoya Restrepo et al., 2005). Also some secondary compounds like saponins and cellulose are used in industry (Montoya Restrepo et al., 2005). At the internet site https://www.buywholefoodsonline.co.uk 1kg of white quinoa (grain) is sold at £7.84 (approximately €9). In Portugal 1kg of quinoa grain costs €10 at https://www.continente.pt.

Halimione portulacoides has recently entered the market as a fresh herb used in salads and to spice dishes. It is sold in few online gourmet shops. At the internet site http://www.finefoodspecialist.co.uk *H. portulacoides* is sold at £18.50 (approximately €21) per 500g. Note: prices and monetary conversions are referent to October 2018.

To sum up, the nutritional value of both plants, their already existing market value, and their performance under the tested conditions, show that they are both good candidates for the treatment of marine aquaculture effluents under different production regimes, from semi-intensive to super-intensive.

5. Conclusions

Both halophytes showed to be good candidates for the treatment of marine aquaculture effluents, however with species-specific characteristics. On one hand *C. quinoa* showed a faster growth and better nutrient removal performance when compared to *H. portulacoides*. On the other hand, the latter presented a lower antioxidant content, which might indicate a better adaptation to higher salinities and oligotrophic environments, which are typical of coastal salt-marshes. It should be considered that *H. portulacoides* has a slower growth rate than *C. quinoa* and might never reach a greater biomass, requiring more time to develop, however, it is better suited to more saline IMTA than *C. quinoa*. Nevertheless, *C. quinoa* presents some leverage because it is already well established on the market, however, *H. portulacoides* has untapped potential which gives it an advantage by the novelty factor. Thus, the type of aquaculture regime, regarding nutrients wasted and salinity, must be considered before choosing the plant species to co-cultivate and also consumer acceptance must be studied. It is also important to find the best factors combination in terms of valuable production and plant health. In this work, N100 seems to be the treatment where both plant species had a better performance, concerning biomass gain.

Further research is necessary to fully understand the potential of both plants to be integrated in an IMTA system. The next step would possibly be to narrow down the ideal nitrogen range for the balanced growth of the plants, see the interactions with other abiotic factors, like phosphorus availability and light, and perhaps assess the performance of these plants when these abiotic factors oscillate, as would happen in real conditions.

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